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Determination of ethyl glucuronide in hair samples by liquid chromatography/electrospray tandem mass spectrometry

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A method for the determination of ethyl glucuronide (EtG) in hair samples, using liquid chromatography/electrospray tandem mass spectrometry (LC/ESI-MS/MS), was developed and validated. The treatment of hair samples was as follows: to 100 mg of washed (dichloromethane followed by methanol, 1 ml each) and cut (1–2 mm) material, 700 μl of water, 20 μl of internal standard solution (pentadeuterated EtG, D_5-EtG, 500 μg/l) and 20 μl of methanol were added. Samples were incubated at 25 °C overnight and then ultrasonicated for 2 h. Finally, 8 μl of the centrifuged solution (13 000 rpm) were analyzed by LC/ESI-MS/MS in negative ion mode. The surviving ions of EtG and D_5-EtG were monitored together with the following MRM transitions: *m/z* 221 → 75, *m/z* 221 → 85 (EtG) and *m/z* 226 → 75, *m/z* 226 → 85 (D_5-EtG). The method exhibited a mean correlation coefficient better than 0.9998 over the dynamic range (3–2000 pg/mg). The lower limit of quantification (LLOQ) and the limit of detection (LOD) were 3 and 2 pg/mg respectively. The intra- and interday precision and accuracy were studied at four different concentration levels (3, 5, 56 and 160 pg/mg) and were always better than 7% (*n* = 5). Matrix effects did not exceed 20%. The method was applied to several hair samples taken from autopsies of known alcoholics, from patients in withdrawal treatment, from social drinkers, from adult teetotalers and from children not exposed to ethanol, with EtG concentrations globally ranging from ≤2 to 4180 pg/mg. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: ethyl glucuronide; hair; LC/ESI-MS/MS; ethanol; alcoholism

INTRODUCTION

Alcohol addiction is a major social problem worldwide. The possibility of detecting the use of ethanol for a relatively long period of time after intake could be very useful for monitoring abstinence in a number of cases of clinical and forensic interest (withdrawal treatment, driving license reissue/renewal, minor adoption, divorce proceedings, etc.). Long-term direct markers, such as the ethanol metabolites ethyl glucuronide and fatty acid ethyl esters in hair samples are currently under evaluation for this purpose.

β-D-Ethyl glucuronide (EtG) is the first ethanol direct metabolite that was found in hair in 1993 by Sachs[1] and later by Aderjan *et al.*[2] and Skopp *et al.*[3] This substance is nonvolatile and water soluble and can be detected in body fluids and tissues, such as blood, urine,[4] and hair.[5]

Few methods to determine EtG concentration in human hair have been recently developed. Skopp *et al.*[6] and Jurado *et al.*[5] have published GC-MS methods in 2000 (lower limit of quantification, LLOQ = 5000 pg/mg) and in 2004 (LLOQ = 50 pg/mg), respectively. Another GC-MS method with negative chemical ionization has been published by Yegles *et al.*[7] (LLOQ = 4 pg/mg). Janda *et al.*[8] proposed a LC-MS/MS procedure in 2002 (LLOQ = 102 pg/mg). None of these authors reported full method validation. Moreover, acquisition was carried out by monitoring either one ion with single-stage MS or one transition by tandem MS, thus limiting the reliability of identification and quantification, especially for forensic purposes.

This paper presents a fully validated method for the determination of EtG in hair by LC-MS/MS with ESI source. The method was applied to several real samples and was found to be sensitive enough to quantify EtG also in the hair of social drinkers.

EXPERIMENTAL

Reagents

Ethyl glucuronide (EtG) and D_5-ethyl glucuronide (D_5-EtG) were obtained from Medichem (Promochem, Milan, Italy). Water was purified by filtering deionized water on a Milli-Q Simplicity 185 filtration system from Millipore (Bedford, MA, USA). Formic acid for mass spectrometry was purchased from Sigma-Aldrich (St Louis, MI, USA). HPLC-grade methanol and acetonitrile were obtained from Mallinkrodt Baker (Milan, Italy).

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The mobile phase consisted of a mixture of 0.1% (v/v) formic acid (A) and acetonitrile (B). The aqueous solution was filtered on 0.45-μm PTFE filters (SUN Sri, Duluth, GA, USA). Filters were ultrasonicated for 20 s in acetonitrile before use.

Instrumentation

LC-MS/MS analyses were performed with an Agilent 1100 Series system (Agilent Technologies, Palo Alto, CA, USA) interfaced to a 4000 Q-TRAP (Applied Biosystem/MDS SCIEX, Foster City, CA, USA) with an electrospray (ESI) Turbo V™ Ion Source. The LC instrumentation was composed of a vacuum degasser, a binary pump and a microautosampler maintained at 4 °C. The injector needle was externally washed with water prior to any injection. The Chrompack Inertsil ODS-3 column (100 × 3 mm i.d., 3-μm particle size) equipped with a Chrompack (10 × 2 mm) RP guard column (Varian, Walnut Creek, CA, USA) was kept at 25 °C during the analysis. Chromatographic elution was as follows: constant flow of 0.2 ml/min at 99% A for 8.0 min, rinsing step at 10% A for 2 min and reequilibration at 99% A up to 21 min. A ten port Valco valve (VICI Valco Instruments, Houston, TX, USA) was used to direct the LC flow into the mass spectrometer from 0 to 8.5 min and then switched to waste up to 18 min. Postcolumn addition of acetonitrile (0.1 ml/min) was performed with a second pump (L-6200 Merck-Hitachi, Tokyo, Japan) connected to LC flow with a tee mixer before the mass spectrometer in order to enhance analyte ionization.[4,8] The ESI source settings were ion-spray voltage, 4200 V; source temperature, 400 °C; nebulization and heating gas (air), 40 and 50, respectively. Multiple reaction monitoring (MRM, Table 1) was optimized using nitrogen as collision gas (with pressure set at level 5) and a dwell time of 75 ms. The transitions m/z 221 → 75 and m/z 226 → 75 were used as quantifiers for EtG and D_5-EtG, respectively. Data acquisition and elaboration were performed by the Analyst® software (version 1.4.1, Applied Biosystem/MDS SCIEX).

Sample collection and preparation

Hair samples were collected with scissors from the posterior vertex as close as possible to the skin and the proximal segment (3–5 cm long) was submitted to the following preparation.

Samples (100 mg) were washed with dichloromethane (1 ml, vortex mixed for 30 s, ultrasonicated for 10 min). The solvent was removed with a Pasteur pipette, and a further washing with methanol (1 ml, vortex mixed for 30 s) was performed. After complete removal of methanol, the hair was dried at room temperature with a gentle nitrogen flow. The material was then cut with scissors (1–2 mm), and 700 μl of water, 20 μl of methanol and 20 μl of internal standard aqueous solution (0.5 mg/l) were added. These solutions were incubated at 25 °C overnight and then ultrasonicated for 2 h. Finally, samples were centrifuged at 13 000 rpm for 10 min and 8 μl of the supernatant was injected in the LC/ESI-MS/MS system.

Table 1. MRM parameters

	Q1 mass (amu)	Q3 mass (amu)	Collision energy (eV)	Cell exit potential (eV)
EtG	221.1	75.1	−21	−11
	221.1	85.1	−27	−14
	221.1	221.1	−14	−10
D_5-EtG	226.1	75.1	−29	−12
	226.1	85.1	−25	−13
	226.1	226.1	−14	−9

VALIDATION

Calibration standards and quality control (QC) samples

Both EtG and D_5-EtG standards were prepared by dissolution of each compound in methanol at the concentration of 1 mg/ml. D_5-EtG working solution (500 μg/l in water) was subdivided into aliquots of 3 ml for storage. EtG working solutions were prepared in methanol at the concentrations of 0.015, 0.025, 0.05, 0.1, 0.2, 0.5, 1, 5 and 10 mg/l by independent dilution and subdivided into aliquots of 20 μl. QC samples were prepared from different sources by a different operator by independent dilution at four levels: 0.015, 0.025, 0.28 and 0.8 mg/l. All standard solutions were stored at −20 °C.

Selectivity

Eleven blank hair samples from nine adult teetotalers and two children were analyzed for possibly interfering peaks during the early validation phase.

Linearity of calibration

To an aliquot of blank hair (100 mg), 700 μl of deionized water, 20 μl of internal standard solution (0.5 mg/l) and 20 μl of appropriate EtG working solution were added. Five replicates for each calibration level (3, 5, 10, 20, 40, 100, 200, 1000 and 2000 pg/mg) were analyzed. The calibration curve was estimated by least-squares regression procedure.

Precision and accuracy

Intraday precision, expressed as the relative standard deviation (RSD), was calculated analyzing the QC samples (3, 5, 56 and 160 pg/mg) in five replicates, while interday precision was measured by analyzing the QC samples in duplicate on 5 different days over a 3-week period. The concentration of the analytes in the QC samples was calculated *versus* the daily calibration curves. Accuracy was determined for both analytes as the percentage deviation of the average of results from the corresponding nominal value.

Matrix effects

Hair samples from seven different blank sources and water were spiked at four levels (10, 40, 140 and 200 pg/mg), processed with the described procedure, and EtG absolute peak area in hair and in water were compared. Experiments were carried out in triplicate. Results were calculated as the percentage of the mean deviation of EtG response in hair samples from the response measured in water at the same concentration level.

Process stability

EtG stability during overnight incubation and ultrasonication was evaluated by processing two sets of blank hair samples spiked at different levels (0, 3, 5, 56 and 160 pg/mg, three replicates each). The first set was spiked before incubation, as described in the usual preparation. The second set was treated with the same procedure, except for EtG addition, which was performed after ultrasonication. D_5-EtG stability was carried out as described for EtG, at the same concentration level added to routine samples.

Reproducibility

Several hair samples from autopsies of known alcoholics found positive for EtG in hair were collected together and a homogeneous sample of washed and cut hair was created. This sample was analyzed over a 5-week period.

Proof of applicability

The analysis was applied to real hair samples obtained from 2 children, 9 adult teetotalers, 21 social drinkers, 22 patients in withdrawal treatment, and 8 autopsies.

Optimization of sample washing

Before carrying out the validation procedure, the sample washing was optimized. In particular, possible EtG loss during hair pretreatment was studied measuring EtG concentration in washing solvents. Initially, 6 ml of dichloromethane was added to 600 mg of EtG-positive hair, and the sample was ultrasonicated for 10 min. The solvent was collected, and three aliquots (1 ml) were evaporated to dryness, redissolved in 200 µl of mobile phase and injected in the chromatographic system. To the solid portion, 6 ml of methanol was added, and the sample was vortex mixed for 30 s. Three aliquots (1 ml) of methanol were treated as described for dichloromethane and injected in the LC/ESI-MS/MS system. The sample hair was cut and analyzed as explained above. The mean absolute peak area of the sample was compared to washing solvent response.

RESULTS AND DISCUSSION

Chromatography

EtG and D_5-EtG eluted in about 4 min (Fig. 1, EtG concentration – 434 pg/mg). The total run time, including LC column washing and equilibration, was 21 min. Another ethanol metabolite, ethyl sulfate (EtS), was initially studied using this method. Interfering peaks showing the same retention time and the same 3 MRM transitions (m/z 125 → 125, m/z 125 → 97, m/z 125 → 80) as EtS were observed in hair samples of teetotalers and social drinkers, with no apparent correlation with ethanol consumption.

Validation

No interfering peaks were observed in chromatograms of hair samples from children and teetotalers (Fig. 2). The calibration curve was linear from 3 to 2000 pg/mg and a least-squares regression model ($y = ax + b$, where y is the peak area ratio and x is the concentration of the analyte expressed in pg/mg) was used to fit the whole range. Slopes, intercepts and correlation coefficients are shown in Table 2.

Table 2. Calibration results

Date	Slope (a)	Intercept (b)	Correlation coefficient R^2
27/05/05	0.0107	0.0069	0.9999
31/05/05	0.0100	−0.0231	0.9997
06/06/05	0.0113	−0.0099	0.9999
08/06/05	0.0117	0.0152	1.0000
15/06/05	0.0101	0.1040	1.0000
Mean	0.0108	0.0186	0.9999
Standard deviation	0.0008	0.0500	0.0001

Table 3. Accuracy and precision data

EtG nominal concentration (pg/mg)	Mean calculated concentration[a] (pg/mg)	Accuracy[a] (%)	Intraday precision[b] (%)	Interday precision[b] (%)
3	2.95	1.6	6.87	4.48
5	5.12	2.4	2.39	4.31
56	57.44	2.7	3.67	3.81
160	163.80	2.5	2.38	1.90

[a] $n = 10$.
[b] $n = 5$.

Four concentration levels (3, 5, 56 and 160 pg/mg) over the linear dynamic range were used to evaluate the method's accuracy and precision. Accuracy was constantly better than 3%. Intra- and interday precisions were always better than 7 and 4.5%, respectively (Table 3). The ratio between the peak area of transition m/z 221 → 85 and the peak area of m/z 221 → 75 at the concentration of 3 pg/mg ($n = 14$, samples analyzed on 10 different sessions) was measured by manual integration. The mean RSD of the ratio (221 → 85)/(221 → 75) was found to be better than 5%. On the basis of these results, 3 pg/mg level was chosen as the LLOQ, and relative chromatograms are shown in Fig. 3. A limit of detection (LOD) of 2 pg/mg was calculated (S/N ratio better than 3).

Despite the very simple sample preparation and the absence of a purification procedure, matrix effects were limited to less than 20% as shown in Table 4.

No EtG degradation during sample processing was observed comparing blank hair samples spiked before and after incubation and ultrasonication. In addition, no degradation of D_5-EtG during hair processing was observed (Table 5).

The method allowed the detection of EtG in a number of hair samples, including samples from autopsies of known alcoholics (EtG concentration from ≤LOD to 4180 pg/mg), from patients in withdrawal treatment (EtG from ≤LOD to 434 pg/mg) and even from social drinkers (Fig. 4, EtG concentration, 4.2 pg/mg; EtG concentration in social drinkers, from ≤LOD to 35.4 pg/mg).

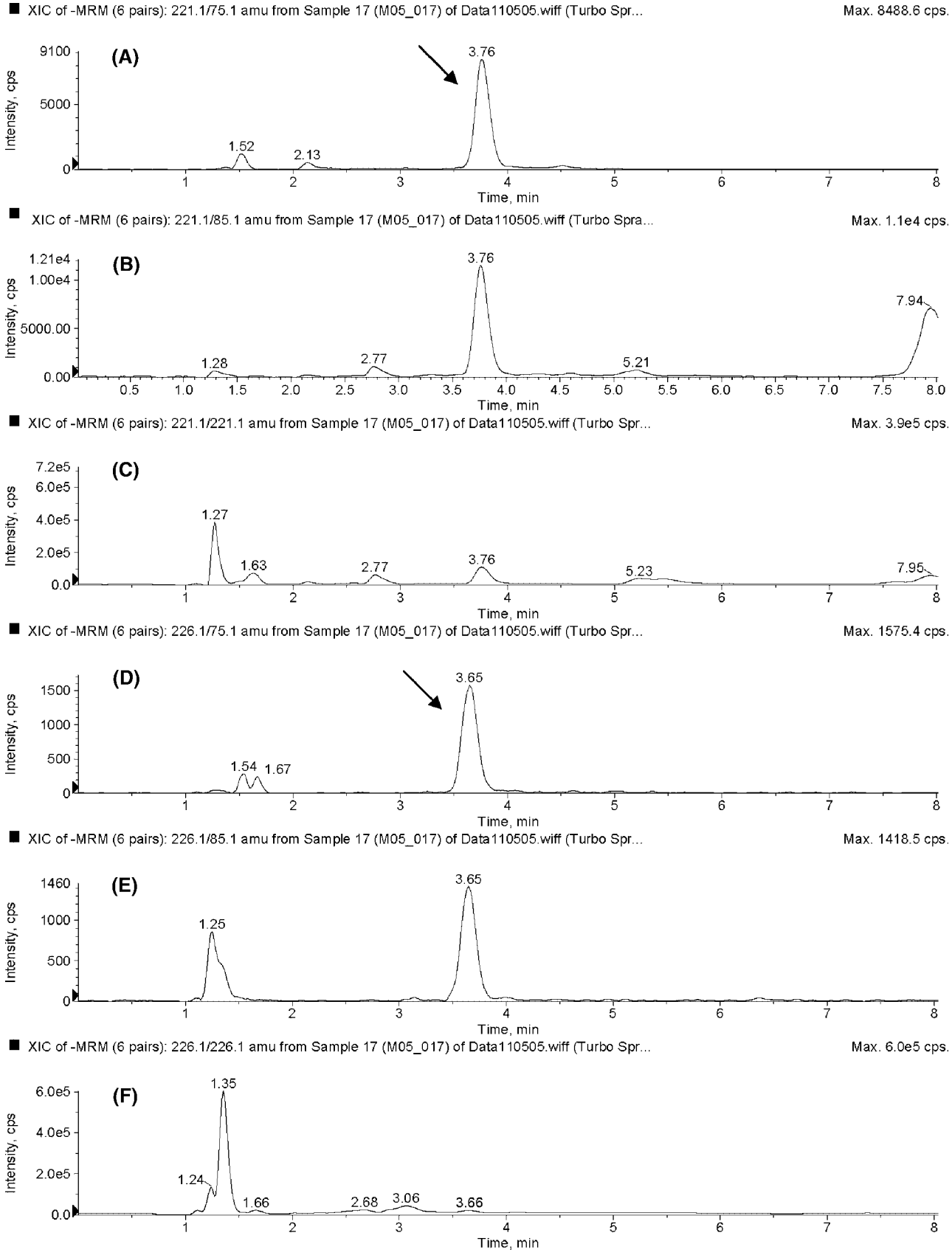

Figure 1. Chromatogram of a real positive hair sample from a withdrawal patient: *m/z* 221.1 → 75.1 (A), 221.1 → 85.1 (B), 221.1 → 221.1 (C), 226.1 → 75.1 (D), 226.1 → 85.1 (E), 226.1 → 226.1 (F). Concentration of EtG was 434 pg/mg.

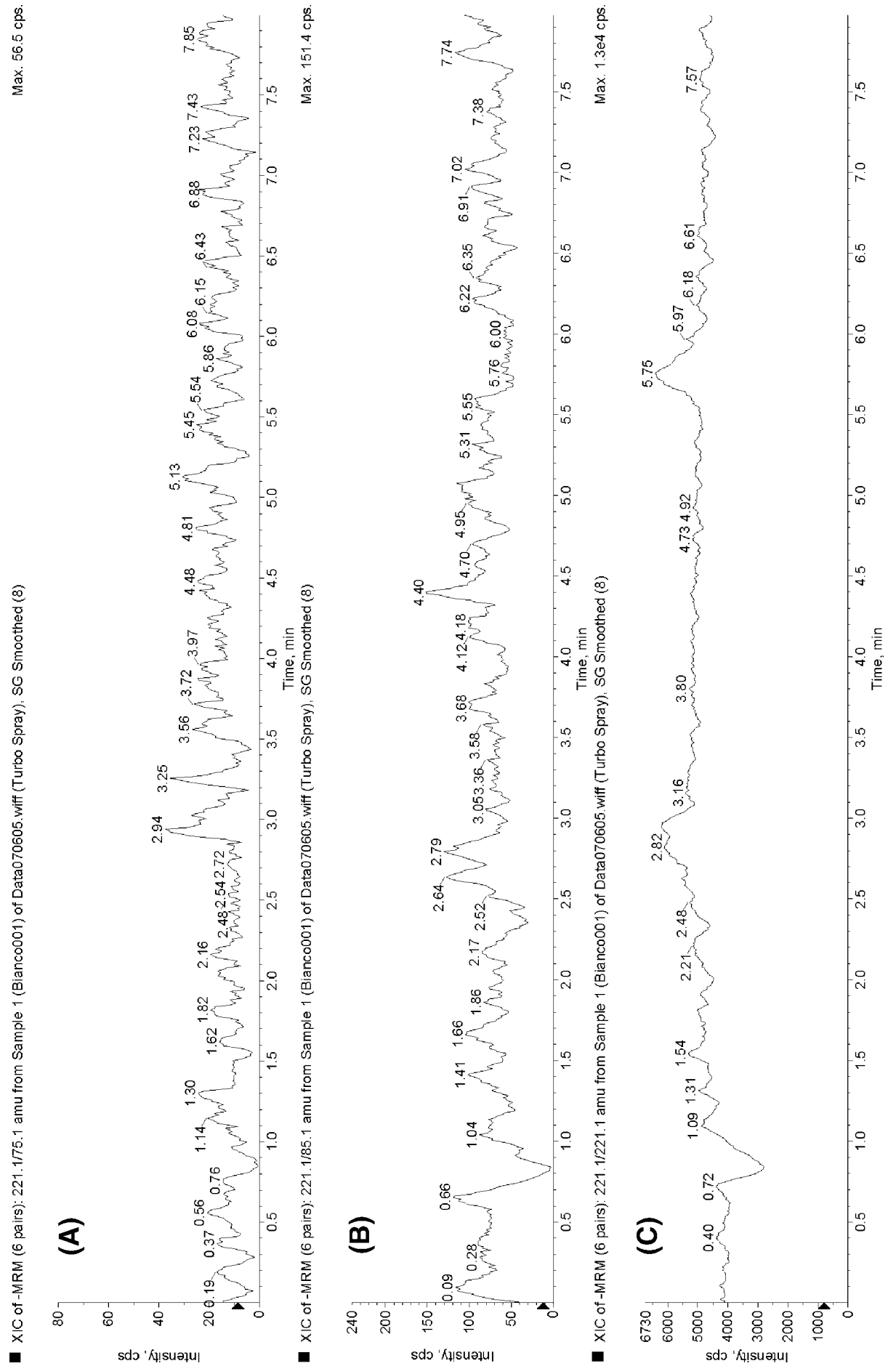

Figure 2. MRM chromatograms *m/z* 221.1 → 75.1 (A), 221.1 → 85.1 (B), 221.1 → 221.1 (C) of EtG in a blank hair sample.

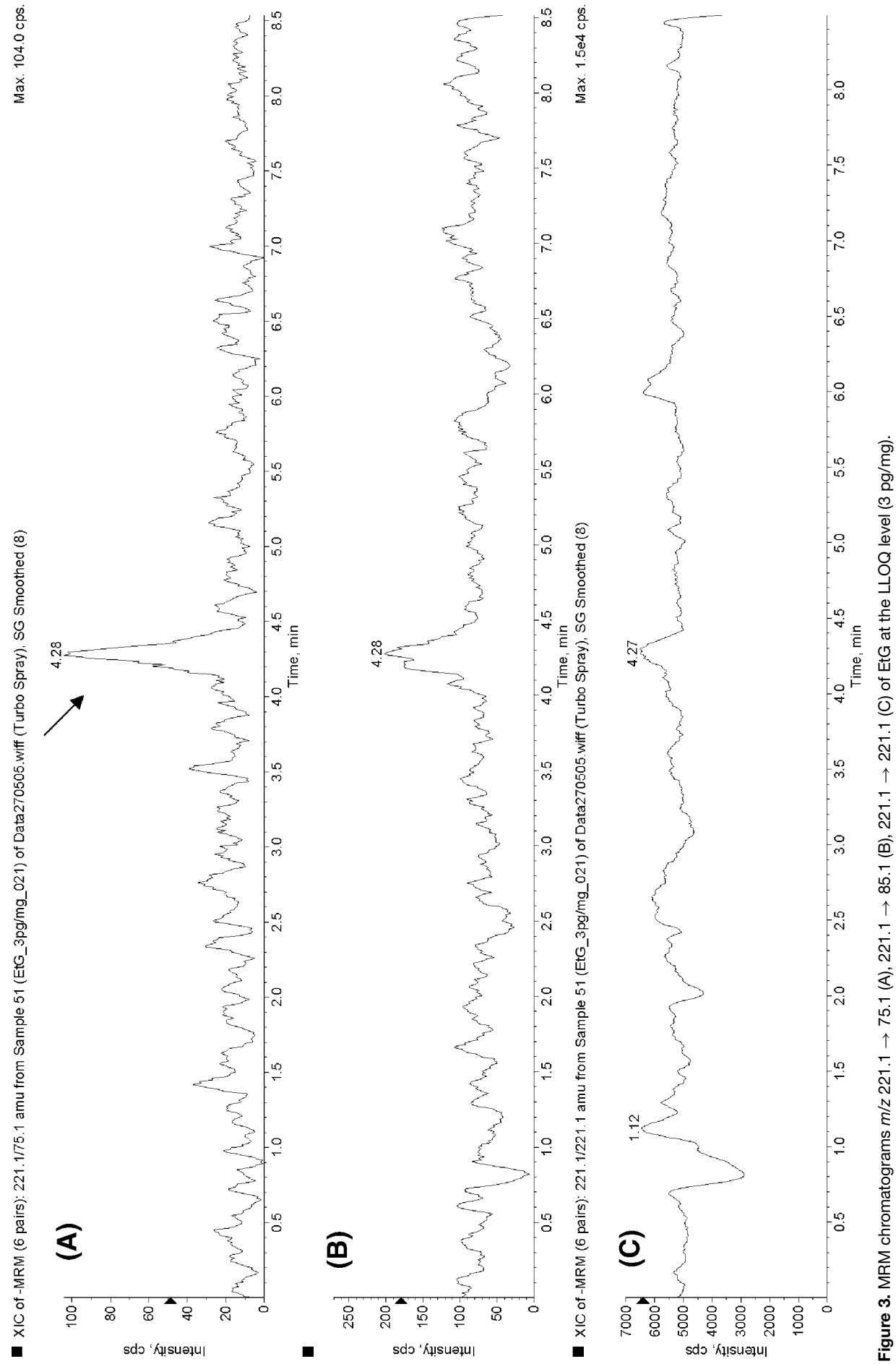

Figure 3. MRM chromatograms *m/z* 221.1 → 75.1 (A), 221.1 → 85.1 (B), 221.1 → 221.1 (C) of EtG at the LLOQ level (3 pg/mg).

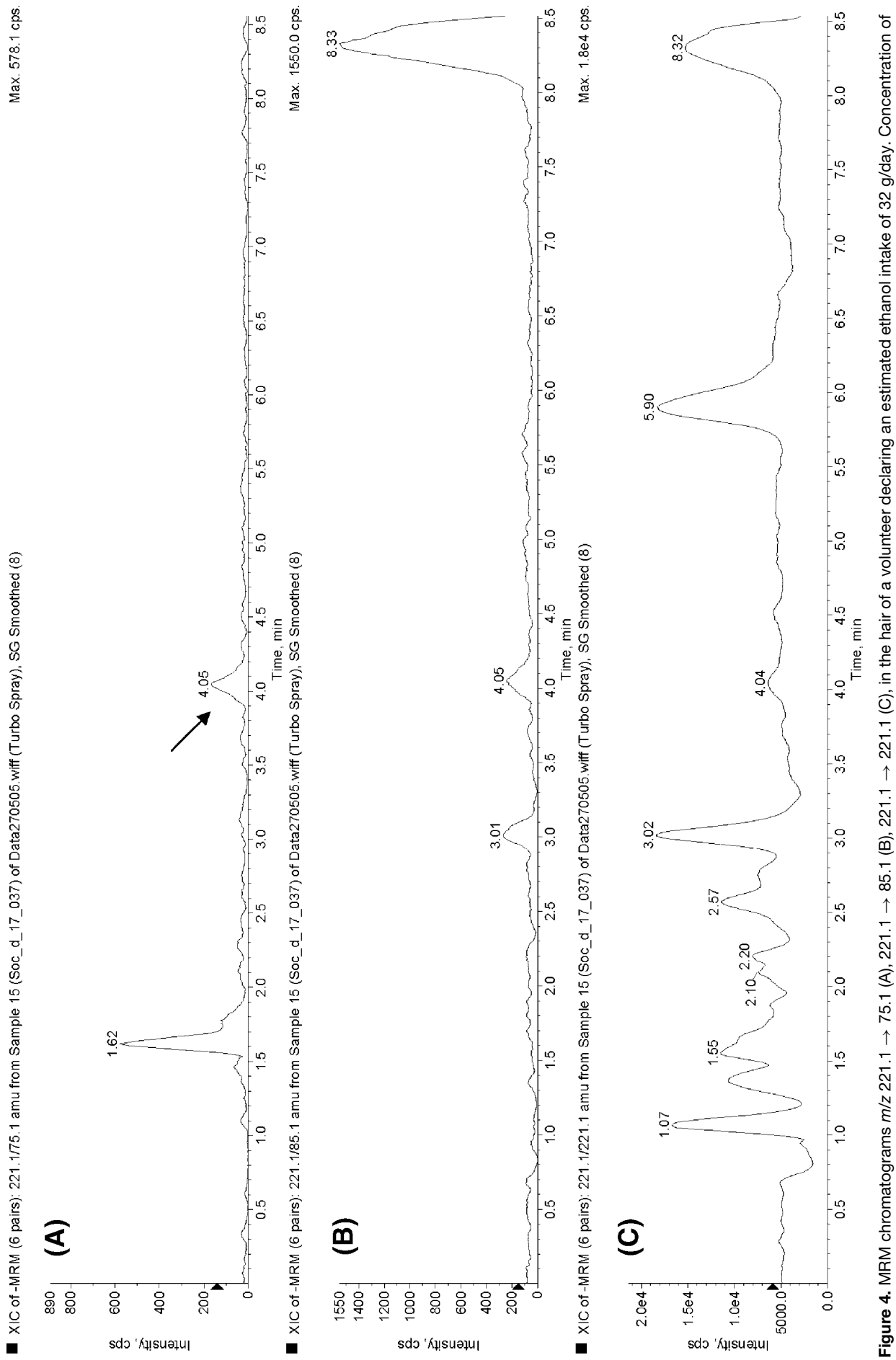

Figure 4. MRM chromatograms *m/z* 221.1 → 75.1 (A), 221.1 → 85.1 (B), 221.1 → 221.1 (C), in the hair of a volunteer declaring an estimated ethanol intake of 32 g/day. Concentration of EtG was 4.2 pg/mg.

Table 4. Matrix effect

Analyte concentration (pg/mg)	Mean relative deviation (%)[a] ($n = 5$)	Standard deviation
10	6.10	19.86
40	−3.66	7.11
140	2.25	13.53
200	0.81	8.73

[a] Calculated as the percentage of the mean deviation of analyte response (absolute peak area) in five different sources of blank hair from analyte response in water.

Table 5. EtG and D_5-EtG stability during sample treatment

Analyte	concentration (pg/mg)	Before incubation and ultrasonication		After incubation and ultrasonication	
		Peak area	Standard deviation (%)	Peak area	Standard deviation (%)
EtG	3	332	5.2	294	6.9
	5	637	6.7	588	8.8
	56	7190	2.5	7180	2.2
	160	22 500	1.2	20 400	1.0
D_5-EtG	100	1027	1.5	1031	4.7

Moreover, EtG concentration was found to be constant in a pool of EtG-positive hair analyzed every 5 days for a 5-week period. These results (Fig. 5) demonstrate that not only the analytical method applied on real positive samples is reliable but also that EtG extraction from hair matrix is constant and reproducible over various sessions of work.

Optimization of sample washing

Washing of hair samples was initially performed by the routine hair-testing procedure used in the laboratory (i.e. 1-ml methanol, 30-s vortex mixing).[9] Yet, chromatograms showed interfering peaks at the analyte's retention time, and matrix effects were significant in several samples. Therefore, a dichloromethane washing was added before methanol in order to remove the lipid component of the hair matrix before extraction. This additional washing resulted in very clean final extracts and much cleaner mass chromatograms.

EtG loss during the washing procedure was found to be negligible, the percentage of EtG lost being 2.332% (±0.224) and 0.219% (±0.001) for methanol and dichloromethane, respectively.

CONCLUSIONS

An LC/ESI-MS/MS procedure for the detection and quantification of EtG in hair samples was developed and fully validated. The method was found to be sensitive enough to quantify 3 pg/mg using 100 mg of sample. Two MS/MS transitions of the precursor ion (and the surviving ion) guarantee selectivity of detection. Probably owing to the efficiency of sample washing, matrix effects were found to be within acceptable limits.

Although the extent of analyte extraction (recovery) from keratin matrix during incubation is still an issue in hair analysis,[5,9] EtG extraction was found to be consistent on five different sessions of work using this procedure.

Finally, the better selectivity of the method as compared to previously published methods, in addition to the notable sensitivity, may provide a better understanding of the correlation between ethanol intake over the medium term and EtG concentration in hair.

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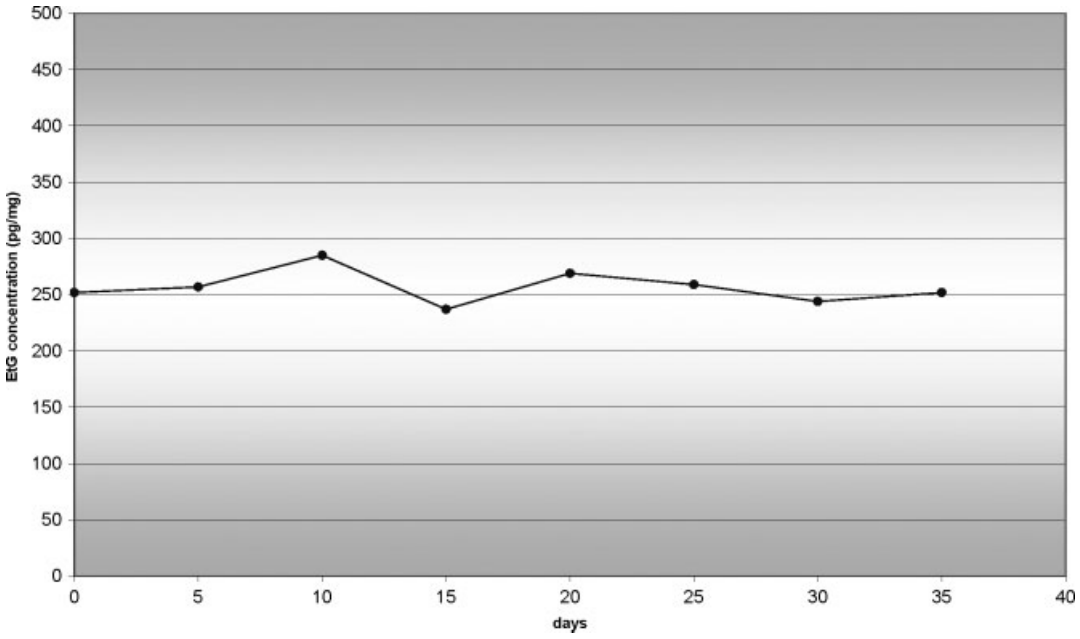

Figure 5. EtG concentration in a pool of hair collected from autopsies of known alcoholics analyzed over a 5-week period.

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